Differential Regulation of the Let-7 Family of MicroRNAs in CD4+ T Cells Alters IL-10 Expression


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MicroRNAs (miRNAs) are ~22-nt small RNAs that are important regulators of mRNA turnover and translation. Recent studies have shown the importance of the miRNA pathway in HIV-1 infection, particularly in maintaining latency. Our initial in vitro studies demonstrated that HIV-1–infected HUT78 cells expressed significantly higher IL-10 levels compared with uninfected cultures. IL-10 plays an important role in the dysregulated cytotoxic T cell response to HIV-1, and in silico algorithms suggested that let-7 miRNAs target IL10 mRNA. In a time course experiment, we demonstrated that let-7 miRNAs fall rapidly following HIV-1 infection in HUT78 cells with concomitant rises in IL-10. To show a direct link between let-7 and IL-10, forced overexpression of let-7 miRNAs resulted in significantly reduced IL-10 levels, whereas inhibition of the function of these miRNAs increased IL-10. To demonstrate the relevance of these results, we focused our attention on CD4+ T cells from uninfected healthy controls, chronic HIV-1–infected patients, and long-term nonprogressors. We characterized miRNA changes in CD4+ T cells from these three groups and demonstrated that let-7 miRNAs were highly expressed in CD4+ T cells from healthy controls and let-7 miRNAs were significantly decreased in chronic HIV-1 infected compared with both healthy controls and long-term nonprogressors. We describe a novel mechanism whereby IL-10 levels can be potentially modulated by changes to let-7 miRNAs. In HIV-1 infection, the decrease in let-7 miRNAs may result in an increase in IL-10 from CD4+ T cells and provide the virus with an important survival advantage by manipulating the host immune response.

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MicroRNAs (miRNAs) are ~22-nt small RNAs that are critical regulators of protein expression within cells at a posttranscriptional level. They are mostly transcribed from noncoding regions of the genome and are initially transcribed by RNA polymerase II into a long primary transcript (1) and subsequently processed by Drosha and DGR8 into a precursor miRNA (pre-miRNA), a shorter stem-loop structure (2, 3) with a 2-nt overhang at the 3' end. Pre-miRNAs are transported to the cytoplasm where they are further processed by Dicer, another RNase III endonuclease (4), into a RNA duplex consisting of a guide strand (antisense) and a passenger strand, each with a 2-nt overhang at the 3’ end. The guide strand is incorporated into the RNA-induced silencing complex, whereas the passenger strand is degraded. Binding of miRNAs to relevant mRNA targets at the 3’ untranslated region (3’UTR) region of mRNA via RNA-induced silencing complex leads to mRNA degradation or translational inhibition (5).

IL-10 is a pleiotropic cytokine expressed by a large number of immune cells, including dendritic cells, monocytes/macrophages, neutrophils, mast cells, basophils, NK cells, and a number of T cell subsets, including Th1, Th2, Th9, Th17, and regulatory T cells (Tregs) (6). Plasma IL-10 levels are increased in patients with HIV-1 infection and directly contribute to the abnormal cytotoxic T cell response to this viral infection (7). The regulation of IL-10 occurs at a number of levels within the cell, including at the epigenetic level, via various signal transduction pathways, by a number of transcription factors, and may also be modulated at a posttranscriptional level by miR-106a (8).

There is a growing literature that shows that cellular miRNAs play an important role in HIV-1 pathogenesis (9). To demonstrate the importance of miRNAs in HIV-1 infection, Dicer and Drosha, key components of the miRNA pathway outlined above, have been previously knocked down using small interfering RNAs in HIV-1–infected PBMCs with a marked increase in viral replication noted (10). Likewise, certain miRNAs have been shown to play an important role in maintaining latency in HIV-1–infected resting CD4+ T cells, and these particular miRNAs can also modulate HIV-1 replication kinetics (11).

In this study, we present data clearly demonstrating that let-7 miRNAs are able to modulate the expression of IL-10 at a posttranscriptional level. We show that HUT78 cells (a T cell line)
express high levels of IL-10 and that overexpression of let-7 miRNAs results in a significant decrease in IL-10 levels, whereas blocking these same miRNAs results in a marked and significant rise in IL-10 levels. HIV-1 infection of HUT78 cells results in a fall in let-7 miRNAs with a reciprocal rise in IL-10, suggesting that the decreases in let-7 may play a role in the rise in IL-10 noted with infection. We demonstrate that primary CD4\(^+\) T cells from HIV-1–infected individuals have significantly lower let-7 miRNAs than healthy controls (HC), and we postulate that these changes in miRNA levels may be associated with the increased IL-10 seen in HIV-1 infection. The altered let-7/IL-10 axis observed in HIV-1 infection may play an important role in the dysfunctional cytotoxic T cell response that is known to occur in HIV-1 infection.

Materials and Methods

Ethics statement

Ethics approval to carry out the work presented in this article was approved by the St. Vincent’s Hospital Human Research Ethics Committee. All study participants provided informed written consent prior to blood being drawn.

Patient selection

All HIV-1–infected patients (long-term nonprogressor [LTNP] or chronic HIV-1 infected [CHI]) were antiretroviral treatment naive prior to the samples being taken. To reduce variability in results due to sex differences, all participants studied were male. LTNP samples were obtained from the Australian LTNP cohort that was established in 1994 and is being followed up by the Kirby Institute (formerly the National Centre for HIV Epidemiology and Clinical Research), Sydney, Australia. The LTNPs enrolled in this cohort met the following criteria: asymptomatic HIV-1 disease who had been infected with HIV-1 for at least 8 y prior to study entry and had a CD4\(^+\) T cell count \(\geq 500\) cells/\(\mu\)l in the absence of any antiretroviral treatment (12). For this study we excluded LTNPs who had defective virus ( nef deletion) or HIV-1 coreceptor polymorphisms (homozygous or heterozygous to the delta 32 CCR5 receptor mutation), which may have explained their LTNP status. It is important to note that many of the LTNPs used in the study had detectable viraemia and are not elite controllers. This was deliberate, as we were looking for other factors related to nonprogression for luminescence. The seed sequence refers to nt 2–8 from the 5'-end of the miRNA.

Let-7 mimics

Let-7b seed mutants (either 2 bases or complete scrambled sequence) were synthesized by Life Technologies and co-transfected with the IL10 3' UTR reporter described above. Sequences are described from 5' to 3', as follows: let-7b mimic, antisense strand, UGA GGU AGU AGG UUG UGU GGTG, and sense strand, CCA CAC AAC CUA CCA CUA CATT; let-7b M2 (2-base mutation highlighted in bold), antisense strand, UGA AGU AGG UUG UGG GTTG, and sense strand, CCA CAC AAC CUA CUA CUA CATT; let-7b scrambled (scrambled seed sequence highlighted in bold), antisense strand, UCC ACG GAU AGG UUG UGG GGTG, and sense strand, CCA CAC AAC CUA UCC GUA GATT.

CD14\(^+\) monocyte and CD4\(^+\) T cell isolation and RNA extraction

PBMCs were separated from blood using density centrifugation (Ficoll). CD4\(^+\) T cells and monocytes were separated from PBMCs using magnetic bead technology (Miltenyi Biotec). Initially, CD14\(^+\) cells (monocytes) were positively selected using the magnetically labeled beads and isolated, followed by positive selection and isolation of CD4\(^+\) T cells. Flow cytometric examination of a subset of samples confirmed the purity of magnetically sorted CD4\(^+\) T cells to be \(>98\%\). Following miRNA extraction (QiaGen miRNasy kit), a stringent three-step quality control procedure was followed to ensure only high-quality RNA was used in the arrays. We performed spectrophotometry on the samples, and only RNA that had 260/280 ratios of 1.9–2.0 and 260/230 ratios >1 were accepted. Samples were then analyzed on an Agilent Bioanalyzer chip to confirm RNA purity and to ensure RNA quality was adequate (aiming for samples with a RNA integrity number >7). Lastly, RNA was run on a small RNA chip on the Bioanalyzer to confirm small RNA species were present prior to running the array.

Microarray analysis

In brief, 100 ng total RNA was dephosphorylated, desalted, and labeled with Cy-3 for each sample and then subsequently hybridized to the Agilent Human miRNA Microarray 2.0 G4470B array that contained probes for 723 human miRNAs, and scanned on an Agilent G2505B scanner at the Ramaciotti Centre for Gene Function Analysis, University of New South Wales, according to the manufacturer’s instructions. We used three replicate arrays, each containing eight arrays (n = 24). Agilent TXT files for each array were imported into GeneSpring GX 10 (Agilent), and two samples (one LTNP and one CHI) were outliers by a principal components analysis, had elevated additive error rates, and were thus excluded from...
Let-7 miRNAs MODULATE IL-10 LEVELS IN CD4+ T CELLS

It is well established that IL-10, an important anti-inflammatory cytokine, is elevated in the plasma of HIV-1–infected patients compared with HC (7, 18, 19). We wanted to investigate the regulatory mechanisms behind the observed increase in IL-10 in HIV-1 infection. We first chose to investigate the effect of HIV-1 infection in HUT78 cells, a T cell line. These cells were chosen as they support productive HIV-1 infection (20) and produce high levels of IL-10 even in the absence of HIV-1 infection (21). We measured IL-10 production over time in supernatants of uninfected and infected HUT78 cells using a cytokine bead array. By 24 h postinfection, IL-10 levels were markedly elevated in HIV-1–infected HUT78 cells compared with uninfected cells (Fig. 1A). This difference was most prominent at 72 h (p = 0.008). These results confirmed that, in an in vitro setting, HIV-1 infection is associated with increased IL-10 levels, a finding consistent with the elevated IL-10 observed in plasma from patients infected with HIV-1 (7).

To investigate whether miRNAs played a role in regulating IL-10 levels in HIV-1 infection, we used the in silico screening tool TargetScan 5.1. This online tool demonstrated that the let-7 family of miRNAs possessed a highly conserved 7mer seed match to the 3′ UTR of IL10 (Fig. 1B).

Let-7b and let-7c levels fall rapidly after HIV-1 infection in a T cell model

Using the same in vitro model, we investigated whether let-7 miRNAs were altered following HIV-infection. After infecting HUT78 cells with HIV-1mb, we measured HIV-1 Gag p24 levels in supernatant and found these levels increased significantly at 24 h and were markedly elevated at 72 h (Fig. 2A), consistent with these cells to be infected with the virus. There was downregulation of expression of let-7 family members within 24 h for both let-7b (p = 0.11) (Fig. 2B) and let-7c (p = 0.007) (Fig. 2C) in infected cultures, reaching statistical significance for let-7b at 72 h (p = 0.04). Let-7f levels were not statistically significantly different at any of the time points (Fig. 2D). These results demonstrated that expression levels of let-7 miRNAs fall rapidly following acute infection in an in vitro model, with different let-7 members showing variable expression kinetics. Consistent with our findings, reduced expression of let-7 family members (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, and let-7g) has been previously demonstrated in HeLa cells infected with the HIV-1 clone, NL-A3 (22). As let-7 miRNAs potentially can target the 3′ UTR of IL10, a fall in let-7 miRNAs is therefore consistent with them playing a role in the decreasing levels of IL-10 observed in HIV-1 infection. To explore this hypothesis, however, we needed to manipulate the levels of IL-10.
let-7 miRNAs in HUT78 cells and observe the effect on IL-10 levels.

Modulating let-7 levels directly affects IL-10 levels

Next, we sought direct evidence that manipulation of let-7 levels could impact upon IL-10 expression in HUT78 cells. Overexpression of miRNAs was achieved by transfecting HUT78 cells with let-7 or negative control pre-miRNAs and measuring IL-10 levels in the supernatant. Overexpression of let-7b, c, and f pre-miRNAs (Fig. 3A) alone or in combination reduced IL-10 levels on average by 65% (range 53–83% reduction) compared with negative control pre-miRNA. Overexpression of let-7c did not significantly change IL-10 levels, but there was a strong trend toward reduction (p = 0.06). In the converse experiment, the action of let-7 miRNAs was inhibited using anti-miRNAs with an average 300% increase (range 157–438% increase) in IL-10 levels (Fig. 3B) compared with negative anti-miRNA control. Anti-miRNAs to let-7b failed to show a statistically significant rise in IL-10 (p = 0.06), although there was a strong trend toward increasing IL-10 in these cultures. It was important to check that modulation of let-7 was not affecting IL-10 by changing HIV-1 viral kinetics. To investigate this, we performed experiments measuring p24 in culture supernatant from HUT78 cells overexpressing let-7b, let-7c, and let-7f and compare this to p24 levels in mock-transfected cells. There was no demonstrable change in p24 levels with overexpression of let-7 miRNAs, suggesting that let-7 was not affecting IL-10 levels by changing viral replication (data not shown).

Finally, to determine the specificity of the interaction between the let-7 family and IL-10, we developed a firefly luciferase reporter construct, which contained the \( IL10 \) 3’UTR. Binding of let-7 miRNAs to the \( IL10 \) 3’UTR should theoretically downregulate luciferase expression in this system. HeLa cells were cotransfected with the reporter and either pre-miRNAs or anti-miRNAs against let-7 family members. These experiments showed significant reduction of luminescence in the pre-miR-let-7–treated cells compared with negative miRNA control and empty vector control (Fig. 4A). Furthermore, using let-7 anti-miRNAs to inhibit the action of endogenous let-7 within HeLa cells was associated with an increase in luminescence compared with empty vector and negative anti-miRNA controls (Fig. 4B). Manipulation of let-7 levels in the HUT78 cell line and the \( IL10 \) 3’UTR reporter system in HeLa cells clearly demonstrates that let-7 directly affects IL-10 and that this is occurring through binding of let-7 miRNAs to the \( IL10 \) 3’UTR. Controls including mutated let-7b seed site by 2

![FIGURE 2. Let-7b and let-7c levels fall rapidly following HIV-1 infection. (A) p24 levels are measured in the same time course experiment used to measure IL-10 levels as in Fig. 1 (p24 levels rapidly rise after 24 h; note y-axis logarithmic scale). (B) let-7b levels as measured by qPCR are significantly lower at 72 h compared with baseline, whereas for let-7c (C) they are significantly lower by 24 h (p = 0.007). In contrast, let-7f levels do not appear to significantly change over time (D). Experiments were performed in triplicate, and error bars represent +SEM. Unpaired two-tailed t tests were used for statistical analysis.](http://www.jimmunol.org/)

![FIGURE 3. Modulation of let-7 miRNAs directly alters IL-10 expression levels. (A) HUT78 cells were electroporated with either no pre-miRNAs (mock control), a negative pre-miRNA control (Neg pre-miRNA), pre-let–7b, pre-let–7c, pre-let–7f, or a combination of pre-let–7b, c, and f in equimolar amounts. Supernatants were collected at 72 h, and IL-10 levels were measured. This showed that IL-10 was significantly lowered in HUT78 cells overexpressing let-7 family members (apart from let-7c). (B) Inhibition of let 7 family members increased IL-10 expression. HUT78 cells were electroporated with no anti-miRNAs (mock ctrl), a negative anti-miRNA control (neg anti-miR), anti–let-7b, anti–let-7c, anti–let–7f, or a combination of anti–let–7b, c, and f in equimolar amounts. This resulted in a significant increase in IL-10 levels for all let-7 family members, apart from let-7b. All experiments were performed in triplicate. Error bars represent +SEM. Unpaired two-tailed t tests were used for statistical analysis.](http://www.jimmunol.org/)
bases (M2) or transfection with a scrambled seed sequence did not impact on luminescence demonstrating specificity (Fig. 4C).

Let-7 miRNAs are significantly downregulated in HIV-1 infection

To investigate whether let-7 miRNAs were altered in HIV-1–infected patients, we chose to comprehensively catalog miRNA changes in CD4+ T cells from three distinct patient groups, as follows: eight HIV-1–negative HC, seven patients with chronic HIV-1 infection (CHI), and seven LTNPs (demographics for HIV-1–infected patients are listed in Tables I and II). Total RNA was extracted and run on an Agilent microarray. After normalizing microarray data, we excluded all nonhuman miRNAs, and miRNAs detected fewer than five times, leaving 224 miRNAs de-

Table I. Demographics of LTNPs

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y)</th>
<th>Years after HIV-1 Diagnosis</th>
<th>CD4 Count (/μl)</th>
<th>Viral Load (Copies/ml)</th>
<th>HLA B<em>57 or B</em>27 Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>16</td>
<td>672</td>
<td>&lt;50</td>
<td>B*57</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>16</td>
<td>990</td>
<td>84</td>
<td>—</td>
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<td>18</td>
<td>609</td>
<td>293</td>
<td>B*27</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>15</td>
<td>703</td>
<td>215</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>51</td>
<td>19</td>
<td>552</td>
<td>4,600</td>
<td>B*27</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>10</td>
<td>1,014</td>
<td>7,100</td>
<td>B*57</td>
</tr>
<tr>
<td>7</td>
<td>52</td>
<td>14</td>
<td>1,073</td>
<td>20,800</td>
<td>—</td>
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Table II. Demographics of patients with progressive HIV-1 infection (CHI)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y)</th>
<th>Years after HIV-1 Diagnosis</th>
<th>CD4 Count (µl)</th>
<th>Viral Load (Copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31</td>
<td>2</td>
<td>528</td>
<td>174,500</td>
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<tr>
<td>2</td>
<td>41</td>
<td>6</td>
<td>352</td>
<td>50,400</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>3</td>
<td>456</td>
<td>83,800</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>1 mo</td>
<td>228</td>
<td>420,140</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>1.5</td>
<td>209</td>
<td>24,800</td>
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<tr>
<td>6</td>
<td>33</td>
<td>6</td>
<td>324</td>
<td>144,200</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>7</td>
<td>384</td>
<td>976,730</td>
</tr>
</tbody>
</table>

FIGURE 5. Relative expression heat map of let-7 family members from the three patient groups. There are 10 let-7 family members, and, apart from miR-202, they all show significant downregulation in both CHI and LTNP compared with HC. With regard to the relative expression heat map, high expression is denoted by red, and low levels are depicted in blue, as shown by the color legend at the bottom.
correlate these polymorphisms with disease outcome in both infectious and autoimmune diseases (26). The three best-described polymorphisms found within the IL-10 promoter are as follows: 1) −1082 (A to G transition), 2) −819 (C to T transition), and 3) −592 (C to A transversion) (27, 28). The −592 (C to A transversion) is associated with lower IL-10 production and faster disease progression in late stages of HIV-1 infection (29), whereas the −1082 (A to G transition) is associated with higher levels of IL-10 and slower CD4+ T cell decline and longer survival in HIV-1–infected individuals (30). These studies suggest that IL-10 may have a protective effect in HIV-1 infection. The haplotype −1082A/−592A was associated with faster progression to AIDS in Caucasians but not in African-Americans (31). A South-African cohort study found that individuals with genotypes associated with higher IL-10 production and faster disease progression in late stages of HIV-1 infection (29), whereas the −1082 (A to G transition) is associated with higher levels of IL-10 and slower CD4+ T cell decline and longer survival in HIV-1–infected individuals (30). These studies suggest that IL-10 may have a protective effect in HIV-1 infection. The haplotype −1082A/−592A was associated with faster progression to AIDS in Caucasians but not in African-Americans (31). A South-African cohort study found that individuals with genotypes associated with high IL-10 production (−1082G and −592C) had lower rates of HIV-1 infection, but had higher viral loads during the initial stages of HIV-1 infection (32). As the infection became established, the correlation between genotype and viral load reversed. This implies that the effects of IL-10 on HIV-1 pathogenesis are complex and may be different depending on the length of time the individual is infected. It is very clear that there are multiple ways in which IL-10 is regulated (from promoter polymorphisms to posttranscriptional regulation via miRNAs). Our results suggest a new posttranscriptional mechanism that might further modulate IL-10 levels and that requires consideration when designing studies that look to map the effect of IL-10 on outcomes.

Monocytes are an important source of IL-10, but a recent paper has demonstrated that multiple other cell types contribute to the increased IL-10 in HIV-1 infection, including T cells, B cells, and NK cells (7). This group also demonstrated that elevated IL-10 levels in HIV-1 infection impair HIV-1–specific CD4+ T cell responses. Other studies have suggested that the excess IL-10 may be secreted from a subset of CD4+ HIV-1–specific T cells in HIV-1–infected individuals. The IL-10 from these HIV-1–specific T cells is thought to inhibit the proliferation of other T cells in an Ag-nonspecific manner (33). The elevation of IL-10 in plasma from HIV-1–infected individuals does not allow one to conclude which cells have differentially contributed to the IL-10. Hence, we chose to investigate the effects of modulating let-7 levels on IL-10 expression in a T cell model amenable to manipulation of the miRNA levels without activating the cell. Ideally, we would have liked to manipulate miRNA levels in primary T cells infected with HIV-1, but one needs to activate these cells using anti-CD3 and anti-CD28 to stimulate the cells to allow productive infection. Recent research has demonstrated that T cell activation greatly alters endogenous miRNA levels (34) and hence would bias our results. Furthermore, although CD4+ T cells may contribute less IL-10 compared with other cell types such as
macrophages, increased production of IL-10 by CD4+ T cells may be an important source of the additional IL-10 observed in HIV-1–infected patients compared with uninfected controls, which contributes to HIV pathogenesis.

IL-10 is regulated at a number of different levels within the cell. These mechanisms include signaling pathways (35, 36), binding of a number of transcription factors to the IL10 promoter (37, 38), and, more recently, epigenetic changes that affect chromatin structure at the IL10 locus (39, 40). All these have been described as potentially important transcriptional regulators of IL-10. There also appear to be several levels of posttranscriptional regulation with one miRNA to date described, miR-106a, binding to the IL10 3’UTR (8). Additionally, mRNA-destabilizing motifs in the 3’UTR have also been previously described (41). This highlights the complexity of regulation of this important anti-inflammatory cytokine. In our study, miR-106a was not shown to be differentially regulated between patient groups, and therefore was not a factor that may lead to differential expression of IL-10 between patient groups. However, our results strongly suggest let-7 family members can also regulate IL-10 expression in CD4+ T cells, and their differential expression in CD4+ T cells may thus be associated with altered disease progression. Furthermore, our results demonstrate HIV-1 infection leads to a decrease in let-7 levels and that these changes were associated with a concomitant rise in IL-10 levels. It should be noted that the vast majority of CD4+ T cells from HIV-1–infected patients are not infected with the virus. Hence, the global downregulation in let-7 miRNAs in these cells is likely to be a bystander effect caused possibly by the activating stimulus of an ongoing viral infection. Another possibility is that there is a skewing in the various T cell subsets in HIV-1–infected patients, and differences in miRNA content within these subsets explain differences in miRNA profiles observed between uninfected and infected patients in our study. Recently, it has been demonstrated that T cell subsets display unique miRNA signatures (42), and further work in HIV-1–infected patients will be required to see whether this turns out to be the case in HIV-1 infection.

Let-7b has been recently linked to regulating another cytokine, IFN-β (43) (along with miR-26a, miR-34a, and miR-145). This regulation was demonstrated in macrophages, and there appeared to be a negative loop feedback mechanism between expression of this cytokine and miRNA levels. IL-10 levels have been shown to decrease in the plasma of HIV-1–infected patients who have been treated with highly active antiretroviral therapy (7). Whether or not let-7 miRNAs increase in this population of treated patients is not known, but if let-7 miRNAs normalize with treatment, this would point to a regulatory mechanism directly related to the magnitude of viral load.

This study highlights new areas of research into pathogenic mechanisms of HIV-1 infection. Raised IL-10 in chronic viral infections is believed to impair host immune responses and contribute to viral persistence (44). Targeted alteration of specific miRNAs may also play an important adjuvant role to future vaccine strategies to augment immune responses. Our work shows let-7 miRNAs have the potential to regulate IL-10 at the posttranscriptional level, and this mechanism plays one part in a complex regulatory network controlling IL-10 expression.

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Disclosures

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References


